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(54) Title: TRANSMISSIBLE, MAMMALIAN GENES ASSOCIATED WITH TUMOR METASTASIS

(57) Abstract

A method for detecting a discrete, transmissible, mammalian gene associated with tumor metastasis. Using the method, a gene associated with metastasis and originating in the human cervical carcinoma line ME-180, metastatic to omentum, and a gene associated with metastasis and originating in the human neuroblastoma cell line SK-N-MC, metastatic to the supraorbital area, have been detected and identified.

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TRANSMISSIBLE, MAMMALIAN GENES ASSOCIATED WITH TUMOR METASTASIS

Description

Technical Field

This invention is in the field of molecular biology and more specifically relates to the detection and isolation of discrete, transmissible, mamalian genes associated with tumor metastasis.

Background Art

Although the molecular basis for malignant transformation leading to cancer is not yet fully understood, much information about the process has been developed recently using molecular biology techniques. For example, while it has long been thought that transformation involved the alteration of critical genes, referred to as oncogenes, such discrete oncogenes have only recently been isolated and shown to cause transformation.

One recent approach to isolation of an oncogene involved the transfer of tumor cell DNA from the EJ bladder carcinoma cell line into non-transformed NIH 3T3 mouse fibroblasts. In this work, it was found that the phenotype of cellular transformation could be passed from cell to cell in this manner. Tumor DNA was able to induce foci of transformed cells in the recipient NIH monolayer cultures while DNA from normal, untransformed donor cells failed to produce foci. See Shih, C., Shilo, B., Goldfarb, M.P., Dannenberg, A. and Weinberg, R.A. Proc. Natl. Acad. Sci. USA 76:5714-5718 (1979); Cooper, G.M., Okenquist, S. and Silverman, L. Nature 284: 418-421 (1980); Shih, C., Padhy, L.C., Murray, M.J. and Weinberg, R.A. Nature 290: 261-264 (1981); Krontiris, T.G. and Cooper, G.M. Proc. Natl. Acad. Sci. USA 78: 1181-1184 (1981); and Perucho, M. et al. Cell 27: 467-476 (1981). These results demonstrated oncogenic factors present in the EJ tumor cell line DNA which were apparently absent from the DNA of normal cells.

Studies which examined the sensitivity or resistance of oncogenic DNA from the EJ bladder carcinoma line to treatment of various site-specific endonucleases indicated that certain specific donor DNA sequences were involved in such cellular transformation. See Lane, M.A., Sainten, A. and Cooper, G.M. Proc. Natl. Acad. Sci. USA 78: 5185-5189 (1981); and Shilo, B. and Weinberg, R.A. Nature 289: 607-609 (1981). A discrete, definable oncogene was later directly demonstrated by molecular isolation of a transforming gene from the EJ human bladder

carcinoma cell line by a method involving inter-species transfection. See Shih, C., Weinberg, R.A. (1982) Cell 29: 161-169.

Isolated human sequences from the c-K-*ras* oncogene which are present in certain human lung tumors have been described. See Nakano, H., Yamamoto, F., Neville, C., Evans, D., Mizuno, T., andPerucho, M., "Isolation of Transforming Sequences of Two Human Lung Carcinomas: Strucutural and Functional Analysis of the Activated c-K-*ras* Oncogenes", Proc. Acad. Sci. USA: 71-75, January, 1984; Santos, E., Martin-Zanca, M., Reddy, P., Pierotti, M.A., Della Porta, G., Barbacid, M., "Malignant Activation of a K-*ras* Oncogene and Lung Carcinoma but Not in Normal Tissue of the Same Patient", Science 223: 661-4, February 17, 1984.

Oncogenes, such as those of the *ras* group, are able to induce full tumorigenic conversion of immortalized cells, such as NIH 3T3 mouse fibroblasts. The effects of these *ras* oncogenes on primary embryo fibroblasts are more circumscribed; in these cells, the *ras* oncogene requires the collaboration of a second oncogene, such as *myc* to induce tumorigenicity. See Land, H., Parada, L.F. and Weinberg, R.A. Nature (London) 304: 596-602 (1983); Ruley, H.E. Nature (London) 304: 602-606 (1983). In either case, *ras*-transformed NIH 3T3 cells or *ras*-plus-*myc* transformed embryo fibroblasts, the cells form localized non-metastasizing tumors in immunocompetent hosts. See Land *et al.*, *supra*. This suggests that the ability of such tumor cells to metastasize requires further alterations.

Up until the present time, however, very little was known about what genetic alterations were involved in metastasis.

Disclosure of the Invention

This invention relates to the discovery of a discrete, transmissible, mammalian gene associated with tumor metastasis.

One embodiment of the invention comprises a method for isolating such a discrete, transmissible gene associated with tumor metastasis from the DNA of a mammalian species. In this method, donor DNA from metastatic mammalian tumor cells ("donor DNA") is fragmented into a multiplicity of fragments, at least one of which contains a discrete, transmissible gene of interest because it is associated with metastasis of the tumor. The multiplicity of fragments and a selectable marker are then transmitted into recipient cells capable of phenotypically expressing the presence of the selectable marker as well as the discrete, transmissible gene of interest. The recipient cells are then cultured under conditions which allow phenotypic expression of the selectable marker. Because of such phenotypic expression, recipient cells can be selected which have acquired the gene of interest, in addition to the marker; they may also contain additional DNA sequences from the donor fragments transmitted into the recipient cells, as well as their own endogenous DNA. Recipient cells selected because of phenotypic expression of the selectable marker are introduced into a mammalian host under conditions

such that the introduced cells would not be metastatic in the absence of the acquired gene of interest. These cells are allowed to form a primary tumor at the site of introduction in the mammalian host, and the host is then examined for metastases. DNA is then recovered from cells of any metastases formed in the host or from cultures of cells removed from the metastases. The aforementioned steps are then repeated substituting DNA from the metastases in place of original donor DNA until DNA recovered from a subsequently arising metastasis is found to contain only that portion of the original donor DNA that constitutes essentially a discrete, transmissible, mammalian gene associated with metastasis and a donor marker indicating that this fragment originated from the original donor DNA. DNA recovered from such metastasis is then broken into fragments, at least one of which is a fragment containing essentially only the gene of interest and donor marker, after which the gene of interest is recovered. Such recovery may be, for example, by procedures of gene cloning.

In one embodiment of the invention which has actually been experimentally performed, a gene associated with metastasis of the human cell line ME-180, a human cervical carcinoma metastatic to omentum, has been isolated. The original DNA from this cell line was serially cotransfected into NIH 3T3 mouse fibroblast cells with pSV2neo DNA, a selectable marker. Subcutaneous injection of such cells into immunocompetent mice resulted in an initial metastasis to the lung of one mouse and a

subsequent metastasis to the abdomen of a second mouse. The ME180 gene does not appear to be closely related to the _myc_ oncogene or to the _ras_ oncogenes in that it did not exhibit reactivity in nucleic acid hybridization tests using DNA probes that are specific for the _myc_ or _ras_ oncogenes.

In another embodiment of the invention, which has also been experimentally performed according to the procedures described above, a gene associated with metastasis of the human cell line SK-N-MC, a human neuroblastoma metastatic to the supraorbital area, has been isolated. Subcutaneous injection of such cells into immunocompetent mice resulted in abdominal metastasis in one mouse.

Isolating a mammalian gene associated with metastasis has many desirable consequences. The isolated gene can be compared with closely related sequences in normal DNA and such comparisons should lead to an understanding of what alterations occur to lead to metastasis.

Additionally, protein coded for by the gene can be produced in significant quantity so that it can be studied to understand the metabolic alterations that occur in the cell during the expression of the metastatic trait. This may also lead to insights into methods by which such a gene, gene products or cellular products affected by the gene product could be antagonized or inhibited. It is, of course, expected to lead to sensitive tests for the presence of this gene or products of this gene involving probes for the gene, mRNA transcribed from the gene, or antibodies reactive with products of the gene.

Brief Description of the Figure

The figure is a photograph of a blot illustrating the results of tests for the presence of human ras and myc oncogene sequences in DNA from metastases derived from secondary transfectants of EJ-6-2-Bam-6a cells.

Best Mode For Carrying Out The Invention

Although the experimental work presented below involved detection of a gene associated with metastasis for the ME-180 cell line and a gene associated with the SK-N-MC cell line, any discrete, transmissible mammalian gene associated with metastasis could be detected using the techniques of this invention. A "discrete" gene is one having a contiguous sequence of base pairs located in one block of sequences of definable length. This block may contain regions coding for protein as well as intervening sequence regions which do not encode protein. A "transmissible" gene is one which can be transmitted from cell to cell using gene transfer (transfection) techniques.

Donor DNA containing such a discrete, transmissible gene can be isolated from other host cell constituents by art-recognized techniques. For example, cells grown in culture can be lysed and the viscous lysate can then be extracted with phenol and with chloroform-isoamyl alcohol. DNA can then be precipitated by ethanol precipitation.

The initial donor DNA containing the gene of interest can be fragmented by mechanical or enzymatic methods. For example, it can be passed

through a narrow gauged needle so that it will be fragmented due to shear. On the other hand, DNA molecules might also be fragmented using restriction endonucleases. The important limitation on fragmentation is that at least one fragment results each time the fragmentation is done which contains in intact form all of the gene of interest.

If the fragment containing the gene of interest does not have an associated marker sequence, one must be added. It is possible to establish experimentally a linkage between a gene and a marker. For example, a donor cell may be tagged with copies of cloned DNA sequence, such as a ØX174 bacteriophage DNA fragment. Upon co-transfection, the donor cell DNA fragments and the cloned marker or "tag" DNA fragments become linked in the recipient cell in a randomly alternating co-polymer. See Lowy, I., Pellicer, A., Jackson, J.F., Simas, I.M. G. K., Silverstein, S. and Axel, R. (1980) Cell 22: 817-823.

Alternatively and preferably, the fragment containing the gene of interest will contain a naturally associated donor-specific marker. For example, human DNA contains over 300,000 copies of the Alu sequence interspersed throughout the entire genome which can serve as a specific marker indicating the presence of human DNA segments. Thus, almost every gene is linked closely, i.e., less than 10 kilobases, to a copy of this repeated sequence which can serve as marker. See Houck, C.M., Rinehart, F.D., and Schmid, C.W. (1979) J. Mol. Biol. 132: 289-306.

Since this Alu sequence is not present in mouse DNA, it is species-specific to human DNA. In fact, a molecular clone of the human Alu sequences probe in Southern blots has been used to detect the presence in mouse cells of introduced human oncogenes of bladder and colon carcinoma origin and of promyelocytic leukemia origin. See Murray, M.J., Shilo, B.Z., Shih, C., Cowing, D., Hsu, H.W. and Weinberg, R.A. (1981) Cell 25: 355-361. Each of these, when resolved from the mouse sequence background, was determined to be affiliated with its own characteristic array of human Alu segments. This species-specific marker, described above, is used in order to insure that a gene finally recovered after isolation procedures did in fact originate with the original mammalian DNA known to be endowed with multiple copies of this specific marker.

Independent of this, fragmented donor DNA can be transmitted into cells with a selectable marker which can be phenotypically expressed by the cells. In the work described herein, the selectable marker employed was the plasmid pSV2neo. Subjecting cultures to G418 selection kills all cells except those carrying this plasmid because of cytotoxic effects of the G418 drug, which drug is inactivated in cells that have acquired the pSV2neo gene. See Southern, E.M. and Berg, P. (1982) J. Mol. Appl. Genet. 1: 327-341.

Of course, other selectable markers could be employed such as the Ecogpt marker which confers resistance to the cytostatic effects of the drug mycophenolic acid.

The preferred route for transmitting donor DNA fragments and the selectable marker into cells is transfection, such as calcium phosphate facilitated transfection. DNAs of any sequence or biological origin can be introduced into mammalian cells by transfection. The transfected DNA need not have any sequence homology with the genome of the recipient cell. Co-transfection is the co-mingling of two or more DNAs prior to their being introduced into mammalian cells, such as by the calcium phosphate facilitated transfection procedure.

EJ-6-2-Bam-6a cells were employed as recipients for the genes associated with metastasis in the experiments described herein. Such cells are NIH 3T3 mouse fibroblast cells transformed with the EJ-Ha-ras oncogene, whose presence allows these cells to form tumors which are localized and non-metastatic. Furthermore, these cells contain only the 6.6 kb fragment of the EJ-Ha-ras oncogene, devoid of any Alu sequences. NIH 3T3 cells, and their derivatives, are unusual because they take up and allow efficient stable expression of transfected DNAs.

Another suitable cell line for transfection is known as Rat-1 cell line. Although most cell lines are relatively refractory to transfected DNAs, it is believed that other cell lines will be found which allow expression of transfected genes.

After co-transfected cells have phenotypically expressed the presence of the selectable marker, cells showing such expression are selected. For example, a colony expressing the phenotype can be

physically picked by standard techniques and the cells of the chosen colony can then be used to seed a large scale culture. Those cells which have expressed the selectable marker can be shown to have concomitantly acquired substantial amounts of the donor cell genomic DNA whose presence may not be manifested in phenotypic alterations of these cells that are apparent in monolayer culture.

Such selected recipient cells can then be introduced into a mammalian host under conditions such that these recipient cells would be tumorigenic but not be metastatic unless they have acquired a donor gene associated with tumor metastasis. Subcutaneous injection is one suitable technique, but the experimental work described herein also documents that intramuscular injection, intraperitoneal inoculation as well as injection into the foot pad of mice were suitable also. Intravenous injection was not suitable since otherwise non-metastasizing tumors created metastases when the tumor cells were intravenously injected.

DNA is then recovered from metastatic cells of metastases formed in the host and employed in place of the original mammalian donor DNA for a second round of transfection into the tumorigenic non-metastatic EJ-6-2-Bam-6a cells. In many cases it is preferred to culture cells from the metastasis in order to obtain larger amounts of DNA than might be obtained directly from cells from the metastasis. The preceding steps are repeated, as required. By repeating all of these steps, serial transmission of the gene of interest, together with any donor marker

sequence, are accomplished. When transfection techniques of human DNA into mouse fibroblasts are done, it has been found that two transfections are usually sufficient in order to create a recipient cell which has the donor gene of interest as well as its associated marker, but lacks virtually all other extraneous donor DNA segments, including extraneous donor segments carrying donor specific marker sequences. Although there may be very small amounts of extraneous donor DNA present in addition to the donor gene of interest, such small fractions should not impede expression by the gene of interest, nor the detection of this specific gene of interest by nucleic acid hybridization procedures.

When a cell has been selected which contains essentially only the gene of interest and its associated donor marker, the marker may be employed in order to identify and recover the gene. For example, a marker gene may be a human repetitive DNA sequence of the Alu type such as that which could be detected by use of the BLUR probe; alternatively, the gene of interest could be experimentally linked to a marker gene prior to the first of the two transfections and this marker gene could then be identified by use of a cloned probe that is reactive with this marker gene. Recovery of the gene can be done by creating a genomic library. The gene of interest or a portion of it, may be contained within a phage also carrying a donor specific marker sequence such as an Alu sequence or an experimentally added marker. This phage may be identified

employing standard hybridization procedures and the phage can be amplified, yielding the human DNA segment in cloned form.

Once isolated, the mammalian gene associated with the Alu sequence, such as the mammalian gene expressing the metastatic phenotype, can be further cloned into other suitable recombinant DNA vectors. Such cloning employs fundamental gene splicing techniques, such as those described by Cohen and Boyer in U.S. Patent No. 4,227,224. Suitable recombinant DNA vectors include bacterial plasmids, phages, animal viruses and yeast vectors. In such techniques, hosts would be employed which allow the recombinant DNA vector to multiply.

Using cloning and expression techniques, significant amounts of the proteins coded for by the gene isolated by the method described herein can be produced. Such proteins can be studied and their effects on modifying other cellular constituents and regulating their level and activity can be elucidated. Moreover, such proteins can be used to produce antibodies by standard antibody production techniques. Thus, for producing polyclonal antibodies, such proteins would be employed to immunize a host, such as a rabbit or a rat, and antibodies to the protein would be collected from serum obtained from the host.

Alternatively, monoclonal antibodies could be produced employing cells which produce antibodies to the protein produced by the isolated gene in typical fusion techniques for forming hybridoma cells. Basically, these techniques involve the fusing of

the antibody producing cell with a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and is capable of producing the desired antibody, in this case an antibody to the protein coded for by the isolated gene. The hybrid cells are then cultured under conditions conducive to the production of antibody which is subsequently collected from the cell culture medium. Such techniques for producing monoclonal antibodies have been well described in the literature. See, for example, U.S. Patent Nos. 4,172,124 and 4,196,265 issued to Hilary Koprowski et al., the teachings of which are hereby incorporated by reference.

A significant use for the antibodies produced to such protein is in assays to detect the presence of protein coded for by the isolated gene associated with metastasis. Such assays include immunoassays, such as those radioimmunoassays employing labelled antibodies or viral antigens.

Probes could also be employed for detecting the gene associated with metastasis or mRNA transcribed by said gene. Such probes might comprise, for example, a labelled polynucleotide complementary to at least a portion of a mammalian gene associated with tumor metastasis.

Metastasis may be prevented or dimininshed by employing antibodies capable of neutralizing protein expressed by a mammalian gene associated with metastasis of the tumor. Alternatively, metastasis may be prevented or diminished by antagonizing the

gene associated with metastasis, or by antagonizing any cellular product or intermediate affected by protein expressed by said gene.

This invention will now be further and more specifically described in the following examples.

EXAMPLES

EXAMPLE 1

ABILITY OF NIH 3T3 CELLS AND EJ TRANSFECTANTS TO FORM TUMORS AND METASTASES IN NUDE AND NFS/NCr MICE

The ability of NIH 3T3 cells and an NIH 3T3 subline transformed by the Ha-*ras* oncogene isolated from the EJ human carcinoma cell line was investigated. Isolation of the Ha-*ras* oncogene from the EJ human bladder carcinoma cell line has been described by Shih and Weinberg. See, Shih, C., Weinberg, R.A. (1982) *Cell 29*: 161-169.

The EJ transfectant, termed EJ-6-2-Bam-6-a, was an NIH 3T3-derived tertiary transfectant carrying the EJ-Ha-*ras* bladder carcinoma oncogene. It was obtained by passing DNA by transfection in two serial cycles through NIH 3T3 cells. DNA of the secondary transfectant was cleaved with the restriction endonuclease Bam HI prior to the third cycle of transfection, freeing the Ha-*ras* oncogene from linkage to the human Alu repeat sequences.

The cells were inoculated into either immunocompetent histocompatible mice, which were 6-8 weeks old NFS/NCr mice or immunoincompetent mice, which were NIH nude (nu/nu) mice previously irradiated

with 500 rad. Each mouse was inoculated by injecting subcutaneously into the flanks of the mice 10^6 cells in 0.2 ml phosphate buffered saline.

The results were as follows:

Cells Injected	Mouse Strain	Tumorigenicity	Metastatic Ability
NIH 3T3	nu/nu	0/6	-
EJ-6-2-Bam-6a	nu/nu	6/6	6/6
NIH 3T3	NFS/NCr	0/8	-
EJ-6-2-Bam-6a	NFS/NCr	62/62	1/62.

As can be seen from the above data, subcutaneous inoculation with NIH 3T3 cells into immunocompetent and immunoincompetent mice produced no tumors. Subcutaneous inoculation of NIH 3T3 cells transformed by the Ha-ras oncogene isolated from the EJ human bladder carcinoma cell line resulted in the formation of tumors at the site of inoculation for all inoculated immunocompetent and immunoincompetent mice. All immunoincompetent mice were found to have metastases in the lungs. However, only one of the 62 immunocompetent histocompatible mice having tumors at the site of inoculation had evidence of metastatic spread. This indicates that the immunocompetence of these mice stood as an effective barrier to metastasis.

The ability of the inoculated cells to form tumors at the site of inoculation, termed tumorigenicity, was determined by making a ratio of the number of mice in which tumors grew over the number

of mice inoculated. Metastatic ability of the tumors was rated by making a ratio of the number of mice found to have metastases over the number of mice found to have tumors at the site of inoculation. Metastasis was determined by sacrificing moribund mice up to 6 weeks after inoculation and subjecting them to necropsy.

EXAMPLE 2

METASTATIC ABILITY OF NORMAL CELLULAR DNA AND DNA OF pSV2neo PLASMID TRANSFECTED INTO EJ-6-2-Bam-6-a CELLS

Since it was desired to co-introduce DNA from human metastatic tumors together with the selectable marker pSV2neo plasmid into EJ-6-2-Bam-6a cells, the ability of normal mouse cellular DNA and DNA from pSV2neo plasmid to cause metastasis was first investigated. Some of the procedures were those of Example 1, but others were different as discussed below.

NIH 3T3 cells were cotransfected with normal cellular DNA from NIH 3T3 cells and pSV2neo plasmid DNA following the procedures of Andersson et al. See Andersson, P., Goldfarb, M.P., and Weinberg, R.A. (1979) Cell 16: 63-75. Briefly, 75 ug of NIH 3T3 DNA and 1 ug of pSV2neo plasmid DNA were applied to 7.5 x 10^5 EJ-transformed NIH 3T3 cells, EJ-6-2-Bam-6-a (2 x 10 cm dishes). Cells were split in a ratio of 1:6. The following day, the cultures were subjected to G418 selection, which killed all cells except those carrying the pSV2neo DNA. See South-

ern, E.M. and Berg, P. (1982) J. Mol. Appl. Genet. 1: 327-341. Colonies resistant to G418 were counted 14 days after transfection.

The G418-resistant cells were collected after they grew to form a dense confluent monolayer (approximately 21 days after transfection). Trypsinized cells were washed with phosphate-buffered saline. All of the colonies of an individual culture dish were pooled and one million cells were injected into a single animal. These pooled cells were injected NFS/NCr mice at several sites: subcutaneous (SC); intravenous (IV); intramuscular (IM); foot pad (FP); and intraperitoneal (IP). These results were as follows:

Cells Injected	Route of Inoculation	Tumorigenicity	Metastatic Ability
EJ-6-2-Bam-6a/Neo	SC	160/166	2/160
"	IV	4/8	4/4
"	IM	4/4	0/4
"	FP	4/4	0/4
"	IP	2/6	0/2.

As can be seen, subcutaneous injection led to metastatic spread in only one mouse out of 38 that had primary tumors. This led to the conclusion that introduction of DNAs, such as NIH 3T3 DNA or pSV2neo DNA, via transfection does not itself significantly affect the metastatic ability of the ras-transformed NIH 3T3 cells.

Twelve primary tumors were selected from the 38 mice inoculated and allowed to grow for up to 3 months. Some were left undisturbed, while others were resected either totally or partially. Many tumors grew so large as to penetrate the intraperitoneal cavity, yet all remained encapsulated without evidence of intraperitoneal seeding or distant metastatic spread. This reinforced the conclusion that these ras-transformed NIH 3T3 did not have significantly high rates of metastasis formation when inoculated subcutaneously.

Mice inoculated intramuscularly in the foot pad or intraperitoneally did not form metastases.

While 4 out of 8 mice intravenously injected in the lateral tail vein formed lung metastases without the presence of a primary tumor, this did not bear on subsequent experiments, all of which employed injection by a subcutaneous route.

EXAMPLE 3

METASTATIC ABILITY INDUCED BY HUMAN TUMOR DNA

The ability of genetic information from a metastatic tumor to confer metastatic ability to EJ-6-2-Bam-6a cells was determined. These cells were well suited for such experiments because they had a low background of spontaneous metastasis (Examples 1 and 2) when subcutaneously injected into mice and, being of NIH 3T3 origin, were able to take up efficiently and express exogenous DNA. See Smotkin, D., Gianni, A.M., Rozenblatt, S. and Weinberg, R.A. (1975) Proc. Natl. Acad. Sci. USA 72: 4910-4913.

DNAs from a variety of human metastatic tumor cell lines were applied to cultures of the Ha-ras transformant (EJ-6-2-Bam-6a), using the cotransfection procedure of Example 2 except substituting a variety of human metastatic tumor cell line DNAs for the normal NIH 3T3 cell line DNA. Human metastatic cell lines whose DNAs were employed included: ME-180, human cervical carcinoma, metastatic to omentum; SK-N-MC, human neuroblastoma, metastatic to supraorbital soft tissue; HuTu 80, human metastatic duodenal adenocarcinoma; Capan-1, human pancreatic adenocarcinoma, metastatic to liver; Calu-1, human lung carcinoma, metastatic to pleura; SK-N-SH, human neuroblastoma, metastatic to bone marrow; Hs0895, human melanoma, metastatic to lung; Hs0891, human renal cell carcinoma, metastatic to lymph node; and SK-MEL-5, human melanoma, metastatic to lymph node.

To eliminate those cells from the transfected cultures which had not taken up and fixed donor human tumor DNA, the metastatic human tumor DNA was cotransfected with pSV2neo plasmid and G418 selection was applied to the transfected cultures. All untransfected cells were thus killed which allowed the outgrowth of large numbers of colonies carrying the pSV2neo marker and a substantial amount of concomitantly acquired human tumor DNA.

It was estimated that each of these cotransfected colonies acquired approximately one one-thousandth of a donor tumor cell genome. See Perucho, M., Hanahan, D. and Wigler, M. (1980) Cell

22: 309-317. Thus, several thousand such colonies needed to be tested in order to be sure that at least one carried a single copy donor gene of interest. Each transfection tested yielded 1500 to 4000 G418-resistant colonies among 12 culture dishes. The colonies of each dish were pooled and 1,000,000 cells from such a pool were inoculated subcutaneously into immunocompetent NFS/NCr mice at subcutaneous sites. Metastases were scored 2-6 weeks later by examination of the abdominal and thoracic organs.

The results were as follows:

DNA Transfected	Tumorigenicity	Metastatic Ability
ME-180 + pSV2neo	32/32	2/32
SK-N-MC + pSV2neo	24/24	1/24
HUTu 80 + pSV2neo	23/24	0/23
Capan-1 + pSV2neo	24/24	0/24
Calu-1 + pSV2neo	24/24	0/24
SK-N-SH + pSV2neo	24/24	0/22
Hs0895 + pSVneo	24/24	0/24
Hs0891 + pSV2neo	23/24	0/23
SK-MEL-5 + pSV2neo	24/24	0/24.

As can be seen, a large proportion of inoculated mice demonstrated primary tumor formation. Initially, however, only one exhibited a metastasis. This single mouse, inoculated with primary transfectants containing human ME-180 DNA formed a metastasis to the lung. Subsequently, one additional mouse inoculated with primary transfectants containing human ME180 DNA and one mouse inoculated with primary transfectants containing SK-N-MC DNA formed metastases to the peritoneal cavity.

EXAMPLE 4

SECONDARY (2°) AND TERTIARY (3°) TRANSFECTANTS EMPLOYING DNA FROM THE METASTASIS IN THE PRIMARY (1°) TRANSFECTANT

Experiments were conducted to determine whether the original metastasis derived from the first inoculation of primary transfectants of human ME180 DNA in the single mouse of Example 3 was induced by human DNA sequences acquired in transfection or developed spontaneously and independently of introduced genes. Thus, tests were made to determine whether the metastatic phenotype could be further passed from this initially arising metastasis to other *ras*-transformed NIH 3T3 cells.

DNA was prepared from the original metastasis and cotransfected into Ha-*ras* transformed NIH 3T3 cells (EJ-6-2-Bam-6a) with pSV2neo employing the procedures of Example 3. The culture of these cells was divided into 12 sub-cultures after 1 day and then placed under G418 selection. Nine of the resulting cultures were inoculated into NFS/NCr mice employing the inoculation procedures of Example 3; six mice has large tumors. Upon autopsy, 4 of the 6 mice carried extensive metastases that were detectable 14-21 days after inoculation.

The four cultures yielding these metastatic cells were retested by inoculation into 38 mice. Of these, 35 displayed tumors and 14 of the tumor-bearing mice carried metastases. This led to the conclusion that the metastatic behavior of cells in these 4 cultures was a reproducible phenomenon. Moreover, it appeared that the metastasis-inducing

determinants, which were present in low concentration in the original human ME-180 tumor DNA, were detectable in enhanced amounts in the DNA of the primary transfectants. Such increase in gene dosage is often seen after transfection of a variety of genes, in that sequences present in single copy and the genomes of donor cells are fixed in multiple copies in the genomes of transfected recipients. See Perucho, M., Hanahan, D. and Wigler, M. (1980) Cell 22: 309-317.

DNAs from 2 of the 4 metastases arising from secondary transfectants were cotransfected once again with pSV2neo DNA and a third cycle of transfection and the resulting cultures tested for metastatic ability in NFS/NCr mice. One secondary transfectant yielded DNA which was used to induce a group of tertiary transfectants. These tertiary transfectants induced primary tumors in 18 of 22 mice inoculated and metastasis was observed in 8 of the 18 mice having primary tumors. In the case of the other secondary transfectant, the derived tertiary transfectants resulted in primary tumors in 11 of 12 mice inoculated and metastasis was observed in 3 of the 11 mice having these primary tumors.

Histological examination of the pulmonary and intraperitoneal metastases revealed that these metastatic tumors were fibrosarcomas, as were the primary non-metasizing tumors.

DNA was also prepared from the second metastasis derived from primary transfectants of human ME180 DNA and cotransfected into Ha-ras

transformed NIH 3T3 cells (EJ-6-2-Bam-6a) with pSV2neo employing the procedures of Example 3. The culture of these cells was divided into 12 subcultures after one day and then placed under G148 selection. The cultures were inoculated into NFS/NCr mice employing the inoculation procedures of Example 3. All twelve mice had evidence of tumor formation; six of the twelve mice carried extensive metasases detectable upon autopsy 14-21 days after inoculation.

Similarly, DNA was prepared from the single metasasis derived from primary transfectants of human SK-N-MC DNA and co-transfected into Ha-ras transformed NIH 3T3 cells (EJ-6-2-Bam-6a) with pSV2neo employing the procedures of Example 3. The culture of these cells was divided into 12 subcultures after one day and then placed under G148 selection. The cultures were inoculated into NFS/NCr mice employing the inoculation procedures of Example 3. All twelve mice had evidence of tumor formation; seven of the twelve mice carried extensive metastases detectable upon autopsy 14-21 days after inoculation.

EXAMPLE 5

TESTS FOR THE PRESENCE OF HUMAN ras AND myc ONCOGENE SEQUENCES IN METASTASES

Tests were conducted for the presence of human *ras* and *myc* oncogene sequences in metastases derived from secondary transfectants of EJ-6-2-Bam-6a cells. The results are illustrated in Figure 1.

DNAs were digested with the eudonucleases Xba I (lanes a - c) and Eco RI (lanes d-l) and analyzed by Southern transfer.

The procedures for Southern transfer analysis have been previously described. See Murray, M.J., Shilo, B.-A., Shih, C., Cowing, D., Hsu, H.-W. and Weinberg, R.A. (1981) Cell 25: 355-61. Briefly, whole cell DNA was prepared from cells grown from metastases. Following restriction endonuclease digestion, 10 ug of the DNA of each metastasis were resolved by electrophoresis through a 1% agarose gel in 40 mM Tris (pH 7.9), 50 mM sodium acetate and 1 mM EDTA. After electrophoresis, the DNA was transferred to nitrocellulose by the method of Southern. See Southern, E.M. (1975) J. Mol. Biol. 98: 503-518. The resulting blots hybridized with probes for human repetitive DNA. The filters were incubated with probes specific for the following human genes Ha-*ras* (lanes a-c), Ki-*ras* (lanes d-f), N-*ras* (lanes g-i), *myc* (lanes j-l). The DNAs analyzed here are as follows: a, d, g, j, EJ-6-2-Bam-6a tertiary transfectant derived from EJ bladder carcinoma DNA; lanes b, e, h, k, ME180-2°Met12L, metastasis derived from secondary transfectants of EJ-6-2-Bam-6a cells transfected with ME-180 metastatic carcinoma DNA; lanes c, f, i, l, ME-180 human metastatic cervical carcinoma. Size markers (in kilobases) are indicated on the ordinate.

An Ha-*ras* homologous segment was identified in all metastases derived from the secondary transfectants, confirming the presence of the EJ-Ha-*ras* gene that had been present in the initially used

recipient cells. No other Ha-ras genes had been acquired beyond those that were present in the recipient cells prior to these transfections. The positive reactivity with the PBR322 probe confirmed the presence of the introduced pSV2neo gene.

EXAMPLE 6

TESTING ENDONUCLEASE FRAGMENTS OF DNA FROM METASTASES FOR METASTATIC ABILITY

DNAs from two of the four metastases derived from the secondary transfectants were digested with the restriction endonücleases EcoRI, BamHI, and HindIII. Digested DNAs were religated with genomic NIH 3T3 DNA. The resulting DNAs were then co-transfected with pSV2neo DNA into EJ-6-2-Bam-6a cells and placed under G418 selection. Cells selected were inoculated subcutaneously into NFS/NCr mice (10^6 cells/mouse). Two to six weeks later, mice were examined for metastasis. Four out of 12 mice that formed tumors when injected with transfectants containing HindIII cleaved DNA formed metastases; three out of 11 mice that formed primary tumors formed metastases when injected with transfectants contaning EcoRI-cleaved DNA; none of the twelve mice that formed primary tumors when injected with transfectants containing BamHI-cleaved DNA formed metastases. Thus, both the HindIII and EcoRI-cleaved DNA segments were biologically active.

Industrial Applicability

The invention described herein is useful in detecting and isolating discrete, transmissible mammalian genes associated with tumor metastasis. Detection and isolation of such genes makes it possible to develóp probes for such genes, or mRNA transcribed from such genes, as well as the production of antibodies against proteins encoded by such genes. This allows sensitive diagnostic techniques for metastasis and potential antagonism of such genes, gene products, or cellular products or intermediates affected by such gene products.

Equivalents

Those skilled in the art will recognize or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed within the scope of this invention.

CLAIMS

1. A method of isolating a discrete, transmissible, mammalian gene associated with tumor metastasis, comprising:

a. forming a multiplicity of fragments of donor DNA from metastatic mammalian tumor cells to provide at least one fragment containing a discrete, transmissible, mammalian gene associated with metastasis;

b. exposing non-metastasizing tumorigenic cells capable of phenotypically expressing the presence of a selectable marker to a selectable marker and to said donor DNA fragments under conditions whereby some of said exposed cells will be recipients of said selectable marker and donor DNA fragments;

c. culturing said exposed cells under conditions sufficient to allow phenotypic expression of said selectable marker in those cells which are recipients of said selectable marker and donor DNA fragments;

d. selecting recipient cells which have phenotypically expressed the presence of said selectable marker;

e. introducing said selected recipient cells into a mammalian host under conditions such that said recipient cells would not be metastatic without the presence of an acquired, discrete, transmissible, mammalian gene associated with tumor metastasis;

f. recovering DNA from metastatic cells of metastases formed in said host in response to the introduction of said selected recipient cells;

g. repeating steps (a)-(f) employing DNA recovered in step (f) in place of DNA from said metastatic cells in step (a) until the DNA recovered in step (f) contains donor DNA containing essentially only said discrete, transmissible, mammalian gene associated with metastasis and a donor marker indicating that said fragment originated from the original donor DNA of step (a); and

h. fragmenting DNA recovered finally in step (f) into fragments at least one of which is a fragment containing essentially only said discrete, transmissible mammalian gene associated with metastatis and a donor marker.

2. A method of Claim 1 wherein said discrete, transmissible mammalian gene associated with metastasis is a human gene.

3. A method of Claim 2 wherein said non-metastasizing transformed cells of step (b) are cotransfected with said selectable marker and donor DNA fragments.

4. A method of Claim 3 wherein said selectable marker comprises a DNA sequence necessary for the survival of cells under certain culturing conditions and said certain culturing conditions are employed in step (c).

5. A method of Claim 4 wherein at least the final cotransfection of human donor DNA is made into non-human mammalian cells.

6. A method of Claim 5 wherein said donor marker comprises a DNA sequence present in human DNA.

7. A method of Claim 6 wherein said donor marker comprises an Alu sequence from human DNA.

8. A method of Claim 7 wherein the final transfection is a transfection of the discrete transmissible human gene associated with metastasis and the Alu marker into mouse fibroblast cells.

9. Isolated mammalian gene associated with tumor metastasis.

10. Isolated protein coded for by a mammalian gene associated with metastasis.

11. Antibody against the protein of Claim 10.

12. Antibody of Claim 11 wherein said antibody comprises monoclonal antibody.

13. A probe for a mammalian gene associated with metastasis.

14. A probe for mRNA transcribed from a mammalian gene associated with metastasis.

15. A probe comprising a labelled polynucleotide complementary to at least a portion of a mammalian gene associated with tumor metastasis.

16. A method of assessing the metastatic propensity of a tumor, comprising:
   a. obtaining cells from said tumor;
   b. testing said cells for the presence of a gene associated with metastasis of said tumor or for a product of said gene associated with metastasis of said tumor.

17. A method of Claim 16 employing a probe comprising a polynucleotide complementary to said gene or mRNA transcribed by said gene.

18. In a method of preventing metastasis of a tumor, the improvement of employing an antibody capable of neutralizing protein expressed by a mammalian gene associated with said metastasis.

19. In a method of Claim 18, the improvement wherein said antibody is a monoclonal antibody.

20. In a method of preventing metastasis of a tumor, the improvement of antagonizing a discrete, transmissible mammalian gene associated with said metastasis.

21. The improvement of Claim 20 wherein said antagonism is achieved by using a pharmacological agent.

22. In a method of preventing metastasis of a tumor, the improvement of antagonizing a cellular product or intermediate affected by the gene product of a discrete, transmissible mammalian gene associated with metastasis of said tumor.

23. A method of screening for the presence of a discrete, transmissible, mammalian gene associated with tumor metastasis, comprising:

a. forming a multiplicity of fragments of donor DNA from metastatic mammalian tumor cells to provide at least one fragment containing a discrete, transmissible, mammalian gene associated with metastasis;

b. exposing non-metastasizing tumorigenic cells capable of phenotypically expressing the presence of a selectable marker to said selectable marker and to said donor DNA fragments under conditions whereby some of said exposed cells will be recipients of said selectable marker and donor DNA fragments;

c. culturing said exposed cells under conditions sufficient to allow phenotypic expression of said selectable marker in those cells which are recipients of said selectable marker and donor DNA fragments;

d. selecting recipient cells which have phenotypically expressed the presence of said selectable marker;

e. introducing said selected recipient cells into a mammalian host under conditions such that said recipient cells would not be metastatic without the presence of an acquired, discrete, transmissible, mammalian gene associated with tumor metastasis; and

f. detecting the presence or absence of metastasis in said host in response to the introduction of said selected recipient cells.

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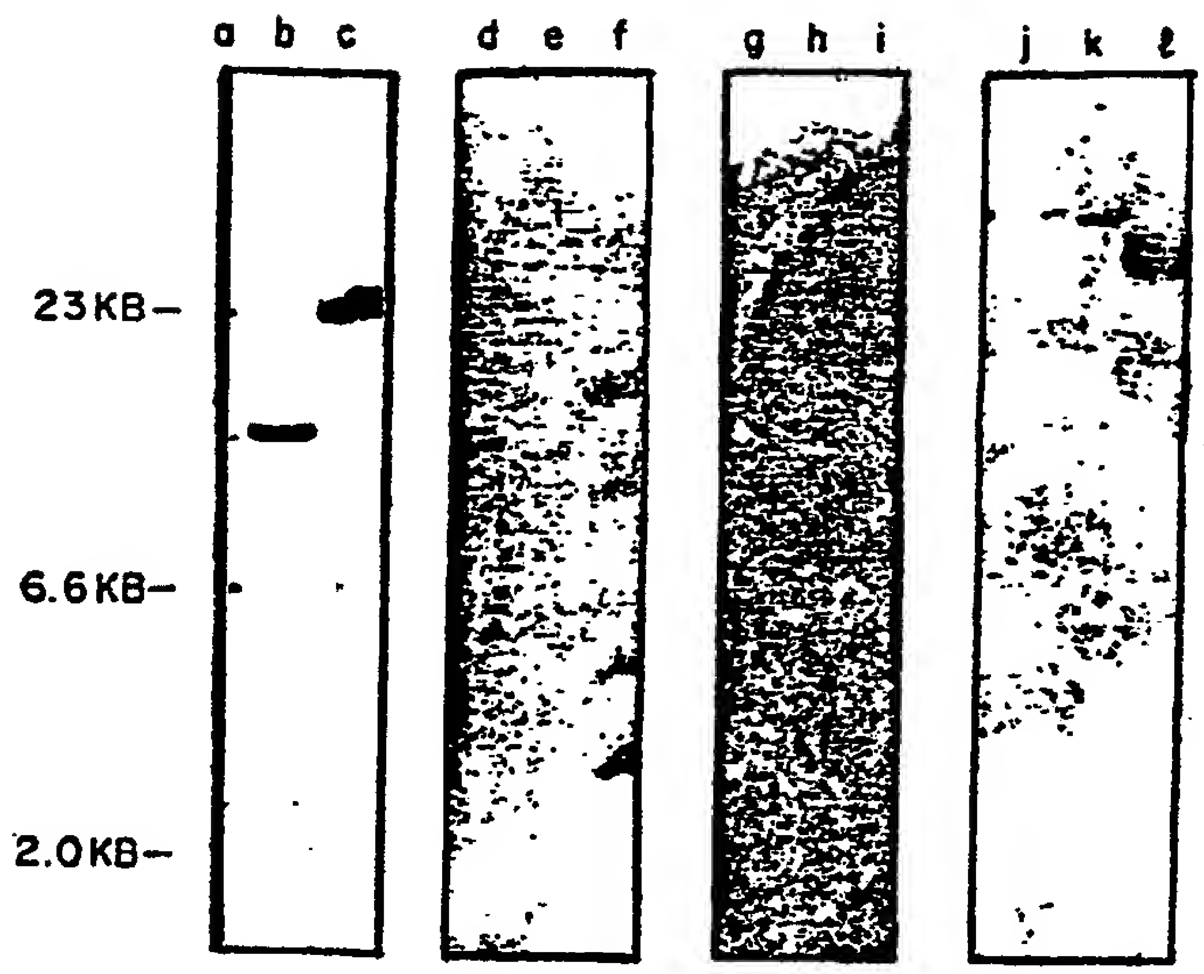

Fig. 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 85/02323

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 12 N 15/00; C 12 Q 1/68; G 01 N 33/574; A 61 K 49/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC^4	C 12 N C 12 Q G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
P,X	Proc. Natl. Acad. Sci. USA, volume 82, no. 6, March 1985 S.C. Bernstein et al.:"Expression of the metastatic phenotype in cells transfected with human metastatic tumor DNA", pages 1726-1730, see the whole article --	1-17,23
A	Chemical Abstracts, volume 101, no. 15, 8 October 1984, Columbus, Ohio, (US) A. Chambers et al.:"Selection for experimental metastatic ability of heterologous tumor cells in the chick embryo after DNA-mediated transfer", see page 510, abstract no. 128204r & Cancer Res. 1984, 44(9), 3970-5 --	1,2,23
A	Biological Abstracts, volume 72, no. 12, December 1981, Philadelphia, (US) B.Y. Klein:"Suggested mechanism for changing tumor cell phenotype. Transfection of host cells with DNA sequences of dead tumor cells", see abstract no. 82074 & Medical Hyptheses 7(5): 645-650 1981	1,2,23

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th March 1986	23 AVR. 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

International Application No PCT/US 85/02323

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Chemical Abstracts, volume 101, no. 17, 22 October 1984, Columbus, Ohio, (US) A.E. Lagarde et al.:"Metastatic properties of distinct phenotypic classes of lectin-resistant mutants isolated from murine MDAY-D2 cell line" see page 499, abstract no. 149108c & Somatic Cell. Mol. Genet. 1984, 10(5) 503-19	1,2,23
A	Nature, volume 304, August 1983, London, (GB) H. Land et al.:"Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes", pages 596-602, see whole article	1-17,23
A	Chemical Abstracts, volume 100, no. 23, June 1984, Columbus, Ohio, (US) K.H. Vousden et al.:"Three different activated ras genes in mouse tumors; evidence for oncogene activation during progression of a mouse lymphoma", see page 145, abstract no. 186643y & Embo. J. 1984, 3(4), 913-17	1,2,23
A	Biological Abstracts, volume 71, no. 11, November 1981, Philadelphia (US) A. Raz et al.:"In vivo isolation of a metastatic tumor cell variant involving selective and nonadaptive processes", see abstract 74914 & J. Natl. Cancer Inst. 66(1): 183-189 1981	1,23

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. [x] **OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE** [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. [x] Claim numbers °°)..... because they relate to subject matter not required to be searched by this Authority, namely:

°°) 18-22 See PCT Rule 39.1(iv) Methods for treatment of the human or animal body by surgery or therapy as well as diagnostic methods

2. [ ] Claim numbers........., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers........., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] **OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING** [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.